WR-074-93

REPORT DOCUMENTATION PAGE

AD-A269 092

2. REPORT DATE	3. REPORT TYPE AND DATES COVERED

4. TITLE AND SUBTITLE
Effect of heat shock, $[Ca^{2+}]$-i, and cAMP on inositol trisphosphate in human epidermoid A-431 cells

5. FUNDING NUMBERS
2
WIIM

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Walter Reed Army Institute of Research
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8. PERFORMING ORGANIZATION REPORT NUMBER

9. SPONSORING / MONITORING AGENCY NAME(S) AND ADDRESS(ES)
U.S. Army Medical Research and Development Command
Ft. Detrick, Frederick, MD 21702-5012

10. SPONSORING / MONITORING AGENCY REPORT NUMBER

11. SUPPLEMENTARY NOTES

12a. DISTRIBUTION / AVAILABILITY STATEMENT
APPROVED FOR PUBLIC RELEASE:
DISTRIBUTION UNLIMITED

12b. DISTRIBUTION CODE

13. ABSTRACT (Maximum 200 words)

The basal levels of inositol monophosphate, inositol bisphosphate, and inositol trisphosphate ($InsP_3$) in A-431 cells incubated in Na^+ Hanks' solution were, respectively, 1.23 ± 0.18%, 0.17 ± 0.03%, and 0.69 ± 0.07% of the total radioactivity in the cell. When cells were heated, $InsP_3$ increased in a temperature-dependent manner related to the duration of heating. The active form of $InsP_3$, inositol 1,4,5-trisphosphate, increased 237 ± 17% after heating (45 °C, 20 min) then returned to baseline within 15 min after the return to 37 °C. The heat-induced increase in $InsP_3$ was not observed in the absence of extracellular Ca^{2+} or with amiloride treatment. Treatment with the nonhydrolyzable GTP analog Gpp(NH)p stimulated that component of the $InsP_3$ increase due to G proteins. U-73122, an inhibitor of PL-C-mediated processes blocked the increase in $InsP_3$ resulting from heat exposure. Both pertussis toxin (30 ng/mL, 24 h), an inhibitor of G inhibitory protein, and cholera toxin (1 μg/mL, 1 h), a stimulator of G-stimulatory protein, increased $InsP_3$ in unheated cells, and heating failed to induce a further increase, suggesting heat activates G proteins. Likewise, 8-Br-cAMP, IBMX, Ro 20-1724, or forskolin increased $InsP_3$ in unheated cells, and heat did not cause an additional increase. The $InsP_3$ increase induced by 8-Br-cAMP was inhibited by removal of extracellular Ca^{2+} or treatment with verapamil, suggesting that an influx of extracellular Ca^{2+} stimulates $InsP_3$ production. These data are the first to suggest that the heat-induced increase in $InsP_3$ results from both an increase in intracellular Ca^{2+} concentration and activation of G proteins, while the cAMP-induced increase is due to a rise in intracellular Ca^{2+} alone. This increase in $InsP_3$ is not related to a heat-induced intracellular acidification.

14. SUBJECT TERMS
inositol phosphate; epithelia

15. NUMBER OF PAGES

16. PRICE CODE

17. SECURITY CLASSIFICATION OF REPORT	18. SECURITY CLASSIFICATION OF THIS PAGE	19. SECURITY CLASSIFICATION OF ABSTRACT	20. LIMITATION OF ABSTRACT

Effect of heat shock, $[Ca^{2+}]_i$, and cAMP on inositol trisphosphate in human epidermoid A-431 cells

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Kiang, Juliann G., and David E. McClain. Effect of heat shock, $[Ca^{2+}]_i$, and cAMP on inositol trisphosphate in human epidermoid A-431 cells. *Am. J. Physiol.* 264 (*Cell Physiol.* 33): C1561-C1569, 1993.—The basal levels of inositol monophosphate, inositol bisphosphate, and inositol trisphosphate ($InsP_3$) in A-431 cells incubated in Na^+-Hanks' solution were, respectively, 1.23 ± 0.18, 0.17 ± 0.03, and 0.69 ± 0.07% of the total radioactivity in the cell. When cells were heated, $InsP_3$ increased in a temperature-dependent manner related to the duration of heating. The active form of $InsP_3$, inositol 1,4,5-trisphosphate, increased 237 ± 17% after heating (45°C, 20 min) then returned to baseline within 15 min after the return to 37°C. The heat-induced increase in $InsP_3$ was not observed in the absence of extracellular Ca^{2+} or with amiloride treatment. Treatment with the nonhydrolyzable GTP analogue 5'-guanylylimidodiphosphate stimulated that component of the $InsP_3$ increase due to G proteins. U-73122, an inhibitor of phospholipase C-mediated processes, blocked the increase in $InsP_3$ resulting from heat exposure. Both pertussis toxin (30 ng/ml, 24 h), an inhibitor of G inhibitory protein, and cholera toxin (1 μg/ml, 1 h), a stimulator of G stimulatory protein, increased $InsP_3$ in unheated cells, and heating failed to induce a further increase, suggesting that heat activates G proteins. Likewise, 8-bromoadenosine 3',5'-cyclic monophosphate (8-BrcAMP), 3-isobutyl-1-methylxanthine, Ro 20-1724, or forskolin increased $InsP_3$ in unheated cells, and heat did not cause an additional increase. The $InsP_3$ increase induced by 8-BrcAMP was inhibited by removal of extracellular Ca^{2+} or treatment with verapamil, suggesting that an influx of extracellular Ca^{2+} stimulates $InsP_3$ production. These data are the first to suggest that the heat-induced increase in $InsP_3$ results from both an increase in intracellular Ca^{2+} concentration and activation of G proteins, while the adenosine 3',5'-cyclic monophosphate-induced increase is due to a rise in intracellular Ca^{2+} alone. This increase in $InsP_3$ is not related to heat-induced intracellular acidification.

inositol phosphate; epithelia

WE REPORTED PREVIOUSLY that heat shock induces an increase in cytosolic free Ca^{2+} concentration ($[Ca^{2+}]_i$) in human epidermoid cells. The increase is derived initially from an influx of external Ca^{2+} followed by a mobilization of intracellular Ca^{2+} stores (18). A second messenger known to mobilize Ca^{2+} from intracellular sources is inositol trisphosphate ($InsP_3$), a product of the receptor-activated hydrolysis of phosphatidylinositol 4,5-bisphosphate mediated by a coupling G protein and a Ca^{2+}-dependent phospholipase C (PLC) (4).

Changes in $InsP_3$ caused by heating can be physiologically significant. The Ca^{2+} mobilization stimulated by $InsP_3$ may trigger a variety of Ca^{2+}-dependent processes (9, 11, 30) that can affect the function and viability of the cell. For example, heat shock protein induction, which provides tolerance to a second heating, ischemic damage, or hydrogen peroxide toxicity, has been shown in mouse mammary tumor cells and A-431 cells to depend on an increase in $[Ca^{2+}]_i$ (13, 19). Furthermore, cell toxicity reactions related to PLC-mediated increases in $[Ca^{2+}]_i$ (2, 10) are functionally similar to the increases in $[Ca^{2+}]_i$ mediated by PLC and $InsP_3$ after heat shock.

The relationship between $InsP_3$ and the cellular response to heat has been examined in some cells but not in human epithelial cells. Exposure of cells to elevated temperature causes a temperature-dependent increase in $InsP_3$. The phenomenon is observed in Chinese hamster ovary cells, mouse BALB/c 3T3 cells (8), human HA 1 cells, rat PC-12 cells, and human HeLa cells (9). A small increase in $InsP_3$ is observed at 41 or 42°C, but there is a two- to threefold increase in $InsP_3$ at 43 or 45°C (9). In those studies a mechanism(s) underlying the heat-induced increase was not described. It is possible that heat shock may directly stimulate PLC (23). On the other hand, heat may increase $InsP_3$ indirectly by promoting an increase in $[Ca^{2+}]_i$ (16, 18) that stimulates PLC.

Other possible mechanisms by which heat may increase $InsP_3$ involve adenosine 3',5'-cyclic monophosphate (cAMP), because heat shock has been shown to cause an increase in cellular cAMP content (21) concurrent with an increase in $[Ca^{2+}]_i$ (18) and an intracellular acidification (20). The relationship between cellular cAMP content and $InsP_3$ is not clear. Pike and Eakes (26) reported that cAMP analogues exert a stimulatory effect on the formation of inositol phosphates in human A-431 cells, whereas in rat neutrophil (15) and in rat kidney slices (25), cAMP-releasing agents inhibit the phosphoinositide turnover.

This study examined the effects of heat shock, $[Ca^{2+}]_i$, and cellular cAMP content on $InsP_3$ levels in human epidermoid A-431 cells, and it provides the first description of a mechanism by which $InsP_3$ is induced by heat. The heat-induced increase in $InsP_3$ results from both an increase in $[Ca^{2+}]_i$ and activation of G proteins, while the cAMP-induced increase is due to a rise in $[Ca^{2+}]_i$ alone.

MATERIALS AND METHODS

Cells. Human epidermoid carcinoma A-431 cells were obtained from American Type Culture Collection (Rockville, MD) and maintained in 75-cm^2 tissue culture flasks (Costar, Cambridge, MA) in a 5% CO_2-95% air incubator at 37°C. The tissue culture medium was Dulbecco's modified Eagle's medium supplemented with 0.03% glutamine, 4.5 g/l glucose, 25 mM *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid (HEPES), and 10% fetal bovine serum. Cells were fed every 3-4 days. Cells from passages 29-40 were used for experiments.

Heat treatment was accomplished by delivering Na^+-Hanks' solution (in mM: 148 NaCl, 4.6 KCl, 1.2 $MgCl_2$, 1.6 $CaCl_2$, and 10 HEPES, pH 7.26 at 37°C) at 40, 45, or 48°C to adherent cells

and placing cells in the same temperature water bath for a specified period of time. Inositol phosphate determinations were begun either immediately after heat treatment or, in the case of inositol phosphate recovery experiments, after heat treatment and selected times of incubation at 37°C. $[Ca^{2+}]_i$ determination procedures were begun immediately after heat treatment. Cells were acidified by exposure to K^+-Hanks' solution (145 mM) containing nigericin (3 μM) and valinomycin (3 μM) for 5 min at pH 6.80 (16).

Cell homogenization was required for experiments with 5'-guanylylimidodiphosphate [Gpp(NH)p], because Gpp(NH)p cannot enter intact cells. For these experiments cells were removed by scraping and resuspended in 1 ml of Na^+-Hanks' solution before sonication. The cell homogenate was incubated with 10 μM Gpp(NH)p for 30 min at 37°C before heating for 20 min at 45°C. The reaction was terminated by the addition of 2 ml of ice-cold 4.5% $HClO_4$.

Inositol phosphates measurement. Cells were grown on six-well tissue culture plates (2×10^6 cells/well) and incubated with *myo*-[^{3}H]inositol (2 μCi/ml, 0.22 nmol/ml) in growth medium for 24 h. They were washed twice with Na^+-Hanks' solution before the experimental treatment. The reaction was stopped by addition of 3 ml of ice-cold 4.5% $HClO_4$-Na^+-Hanks' solution (2:1, vol/vol) to each well. The plate was chilled for 30 min, and cells were removed by scraping. The cell suspension was centrifuged at 2,500 revolutions/min (rpm) for 10 min. The supernatants were prepared for isolation of the [^{3}H]inositol metabolites by adjusting the pH to 8.0 with a solution of 0.5 M KOH, 9.0 mM $Na_2(BO_4)_2$, and 1.9 mM EDTA. The samples were stored at −20°C overnight. After thawing, the $KClO_4$ salts precipitated and were removed by centrifugation at 2,500 rpm for 10 min. One hundred microliters of the supernatant were used to determine the total radioactivity in the sample. The remainder was applied to 1 ml of suspended Dowex AG 1-X8 resin in the formate form (100-200 mesh). The [^{3}H]inositol phosphates were eluted according to the method of Berridge (3). Inositol, glycerophosphorylinositol, inositol 1-monophosphate, inositol 1,4-bisphosphate, and Ins(1,4,5)P_3 were sequentially eluted with water (24 ml), 5 mM disodium tetraborate in 60 mM sodium formate (24 ml), 100 mM formic acid in 200 mM ammonium formate (36 ml), 100 mM formic acid in 400 mM ammonium formate (24 ml), and 100 mM formic acid in 1.0 M ammonium formate (38 ml). Radioactivity was determined by mixing 1 ml of each eluent with 10 ml of Aquasol scintillation cocktail and counting with a scintillation counter.

The $InsP_3$ fractions were acidified, salt extracted by passing over Dowex 50 resin (H^+ form), and freeze dried. The freeze-dried [^{3}H]$InsP_3$ fractions were dissolved in 2 ml of distilled water and filtered through 0.22-μm filters before injection onto a Whatman Partisil 10 Sax column ($H_2PO_4^-$ form) and chromatographed according to modifications of the procedure of Batty et al. (1). The sample was eluted at 1.2 ml/min by a nonlinear gradient consisting first of water for 4.9 min, followed by a gradient ranging from 0.8 to 1.7 M ammonium formate (adjusted to pH 3.7 with phosphoric acid) for another 25 min. The 1.7 M ammonium formate buffer was run for an additional 5 min, and the gradient was then returned linearly to water over 5 min. One-minute fractions were collected, and radioactivity in the sample was determined by mixing 0.5 ml of each fraction with 1.0 ml water and 10 ml Aquasol before counting with a scintillation counter. Standard retention times were determined by running a mixture of 0.025 μCi of D-[*inositol*-2-^{3}H(N)]Ins(1,4,5)P_3, 0.025 μCi D-[*inositol*-2-^{3}H(N)]inositol 1,3,4,5-tetrakisphosphate, and 150 μM ATP in H_2O and monitoring each fraction for both radioactivity and ATP [absorbance at 245 nm (34)]. The respective retention times for inositol 1,3,4-trisphosphate (represented by ATP), Ins(1,4,5)P_3, and inositol 1,3,4,5-tetrakisphosphate were 18, 20, and 32 min (34). Inositol phosphate levels are reported as a percentage of the total radioactivity present in the cells at the time of inositol metabolites extraction.

Cells were not treated with the phosphatase inhibitor LiCl before, during, or after heat shock, because LiCl (10 mM, 10 min) failed to enhance basal levels of inositol phosphates (Table 1), a finding similar to that observed for macrophage-like J774A.1 cells (34).

Intracellular $[Ca^{2+}]$ measurement. Confluent monolayers of cells on glass cover slips (5×10^5 cells/slip) were washed with Na^+-Hanks' solution, then loaded with 5 μM fura-2 acetoxymethyl ester (AM) plus 0.2% pluronic F-127 (to make cells more permeable to fura-2/AM) for 60 min at 37°C. The cells were washed twice with Na^+-Hanks' solution before fluorescence measurements. The leakage rate of this dye and the method to determine $[Ca^{2+}]_i$ in unheated and heated A-431 cells have been published previously (17, 18).

Statistical analysis. All data are expressed as means ± SE. Analysis of variance, Bonferroni's inequality, and Student's *t* test were used for comparisons of groups (29).

Chemicals. *Myo*-[^{3}H]inositol, D-[*inositol*-2-^{3}H(N)]Ins(1,4,5)P_3, *d*-[*inositol*-2-^{3}H(N)]inositol 1,3,4,5-tetrakisphosphate, and Aquasol were purchased from Du Pont-NEN (Boston, MA). Ro 20-1724 [4-(3-butoxy-4-methoxybenzyl)-2-imidazolidinone] was provided by Hoffmann-La Roche (Nutley, NJ). ^{125}I-cAMP and *myo*-[^{3}H]inositol were purchased from New England Nuclear. Other chemicals used were 1-{6-[(17β-3-methoxyestra-1,3,5(10)-trien-17-yl)amino]hexyl}-1H-pyrrole-2,5-dione (U-73122), 1-{6-[(17β-3-methoxyestra-1,3,5(10)-trien-17-yl)amino]hexyl}-2,5-pyrrolidine-dione (U-73343; generously provided by Upjohn, Kalamazoo, MI), ATP, 3-isobutyl-1-methylxanthine (IBMX), pertussis toxin, cholera toxin, forskolin, 8-bromoadenosine 3',5'-cyclic monophosphate (8-Br-cAMP), verapamil, Gpp(NH)p (Sigma Chemical, St. Louis, MO), fura-2/AM, 3,4,5-trimethoxybenzoic acid 8-(diethylamino)octyl ester HCl (TMB-8), nigericin, and valinomycin (Molecular Probes, Eugene, OR). Whatman Partisil 10 Sax columns were obtained from American Scientific Products (Columbia, MD). AG 1-X8 resin in formate form and Dowex 50 W-X8 resin were obtained from Bio-Rad (Richmond, CA).

RESULTS

Effect of heat on inositol phosphates. The basal levels of inositol monophosphate ($InsP_1$), inositol bisphosphate ($InsP_2$), and $InsP_3$ in A-431 cells at 37°C in Na^+-Hanks' solution were, respectively, 1.23 ± 0.18, 0.17 ± 0.03, and 0.69 ± 0.07% ($n = 21$) of the total radioactivity in the cell. Heating A-431 cells above 37°C elevated inositol phosphates. As depicted in Fig. 1, the increases in $InsP_1$, $InsP_2$, and $InsP_3$ were temperature dependent. A significant increase in $InsP_1$ and $InsP_3$ was observed after heating to 42°C (20 min). Exposure to higher temperature induced further increases (Fig. 1). The changes in

Table 1. *LiCl did not affect inositol phosphate turnover*

	−LiCl	+LiCl
$InsP_1$	2.79±0.38	2.32±0.05
$InsP_2$	0.37±0.04	0.30±0.02
$InsP_3$	0.99±0.11	0.80±0.06

Values are means ± SE and are expressed as a percentage of total radioactivity in cells at time of inositol metabolite extraction. Cells were treated with 10 mM LiCl for 10 min in presence of 1.6 mM external Ca^{2+} ($n = 4$). $InsP_1$, inositol monophosphate; $InsP_2$, inositol bisphosphate; $InsP_3$, inositol trisphosphate.

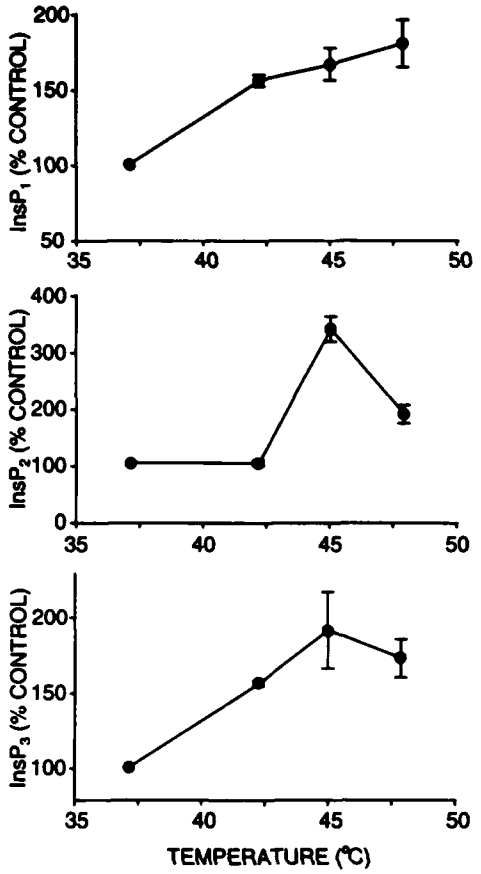

Fig. 1. Effect of temperature on inositol monophosphate ($InsP_1$), inositol bisphosphate ($InsP_2$), and inositol trisphosphate ($InsP_3$). Cells were exposed to 37, 42, 45, or 48°C for 20 min ($n = 4$). Data are expressed as a percentage of those values measured in unheated (37°C) controls.

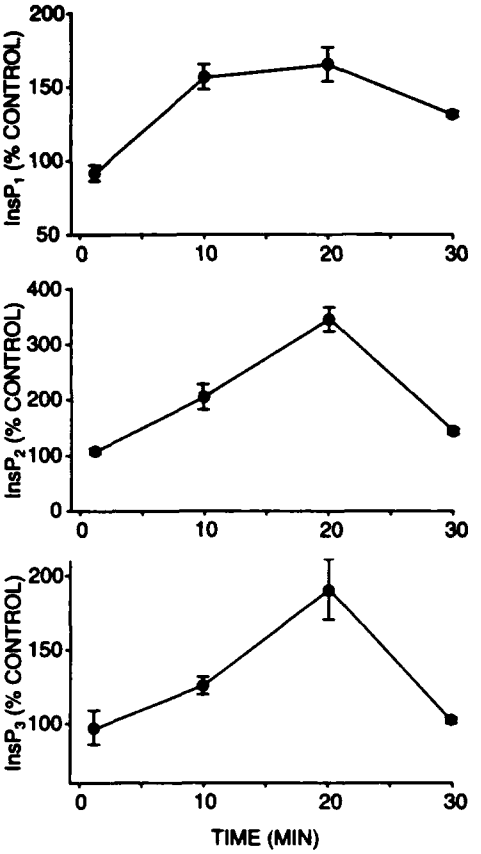

Fig. 2. Effect of duration of heating on $InsP_1$, $InsP_2$, and $InsP_3$. Cells were exposed to 45°C for 1, 10, 20, or 30 min ($n = 4$). Data are expressed as a percentage of those values measured in unheated (37°C) controls.

$InsP_1$, $InsP_2$, and $InsP_3$ also depended on the duration of heat exposure (Fig. 2). Increases were observed after heating cells 10 min at 45°C; maximal increases appeared at 20 min. After heating the cells for 30 min, the level of inositol phosphates diminished, most likely because of a decrease in cell viability (20). Because $InsP_3$ eluates contained $Ins(1,3,4)P_3$ (inactive form), $Ins(1,4,5)P_3$ (active form), and inositol tetrakisphosphates ($InsP_4$), these isomers were further separated using high-performance liquid chromatography. We found that after heat shock $Ins(1,3,4)P_3$ increased slightly, $Ins(1,4,5)P_3$ increased 2.4-fold, and $InsP_4$ decreased 3.6-fold (Table 2).

Inositol phosphate levels in heated cells returned to normal 15 min after heat treatment, and their levels were lower than baseline values 1 h after heating (Fig. 3). Because the basal levels of inositol phosphates often varied between experiments, data from each experiment were compared with their own controls.

Effect of $[Ca^{2+}]_i$ on $InsP_3$. The increase in inositol phosphate was dependent on an increase in $[Ca^{2+}]_i$. In the absence of extracellular Ca^{2+}, basal levels of $[Ca^{2+}]_i$ were reduced from 87 ± 5 to 42 ± 9 nM, and heat treatment did not increase $[Ca^{2+}]_i$ (18). Similarly, the removal of extracellular Ca^{2+} reduced basal levels of inositol phosphates, and no increase was observed after heating (Fig. 4). When cells were heated in the presence of extracellular Ca^{2+} and 100 μM TMB-8, the heat-induced $InsP_3$ increase was unaffected, suggesting that intracellular stores of Ca^{2+} are not involved in elevating $InsP_3$. Previously, we showed 100 μM TMB-8 can attenuate the heat-induced increase in $[Ca^{2+}]_i$ by 50%, because TMB-8 at this concentration blocks bradykinin-induced Ca^{2+} mobilization from an $InsP_3$-sensitive pool. In the absence of extracellular Ca^{2+}, 10 μM bradykinin (33) increased $[Ca^{2+}]_i$ 64 ± 23 nM ($n = 4$) above that of unheated cells not exposed to TMB-8.

We previously demonstrated that the heat-induced increase in $[Ca^{2+}]_i$ is primarily due to activation of the reversed mode of Na^+-Ca^{2+} exchange and is blocked by amiloride (18). If the increase in $[Ca^{2+}]_i$ that is required to increase $InsP_3$ is derived from extracellular Ca^{2+}, then treatment with amiloride should prevent it. Indeed,

Table 2. *Heating increased inositol 1,4,5-trisphosphate and reduced inositol 1,3,4,5-tetrakisphosphate in heated cells*

	Unheated	Heated	Heated/Unheated
$Ins(1,3,4)P_3$	0.043±0.030	0.057±0.014	1.33±0.19
$Ins(1,4,5)P_3$	0.30±0.08	1.01±0.05*	3.37±0.17
$InsP_4$	0.46±0.03	0.10±0.04*	0.22±0.08

Values are means ± SE and are expressed as a percentage of total radioactivity in cells at time of inositol metabolite extraction. Cells were exposed to 45°C for 20 min ($n = 4$ or 5). $Ins(1,3,4)P_3$, inositol 1,3,4-trisphosphate; $Ins(1,4,5)P_3$, inositol 1,4,5-trisphosphate; $InsP_4$, inositol 1,3,4,5-tetrakisphosphate. * $P < 0.05$ vs. unheated cells.

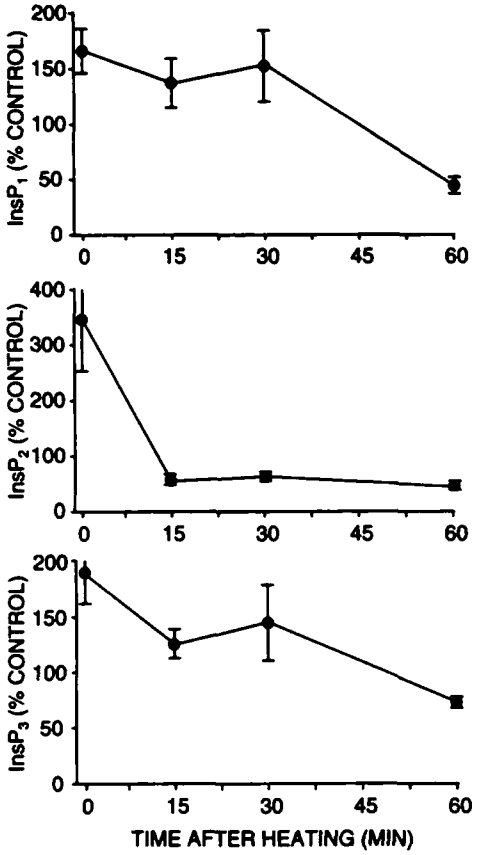

Fig. 3. Recovery of $InsP_1$, $InsP_2$, and $InsP_3$ after heating. Cells were returned to the incubator at 37°C for various times before measurement ($n = 4$). Data are expressed as a percentage of those values measured in unheated (37°C) controls.

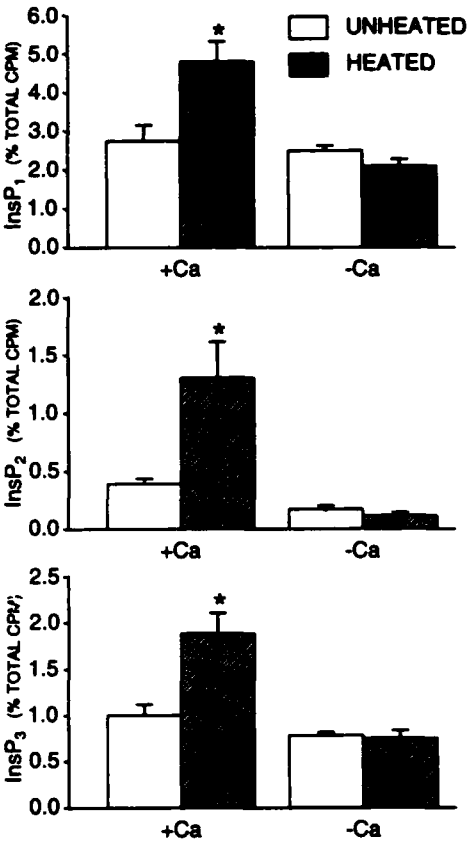

Fig. 4. Dependence of extracellular Ca^{2+} in heat-induced increases in $InsP_1$, $InsP_2$, and $InsP_3$. Cells were heated in Na^+-Hanks' solution either containing 1.6 mM Ca^{2+} (+Ca) or containing 10 mM ethylene glycol-bis(β-aminoethyl ether)-*N,N,N',N'*-tetraacetic acid without Ca^{2+} (−Ca) at 45°C for 20 min ($n = 4$). Data are expressed as a percentage of total radioactivity in cells at time of inositol metabolite extraction. * $P < 0.05$ vs. unheated cells in solution containing 1.6 mM Ca^{2+}. cpm, counts per minute.

amiloride (1 mM) blocked the $InsP_3$ increase (amiloride, 1.32 ± 0.17%; amiloride + heat, 1.52 ± 0.17%; $n = 3$ for each group). These data taken together with data obtained in the absence of external Ca^{2+} support the observation that Ca^{2+} influx is a required element of the $InsP_3$ heat response.

We previously reported that heat shock induces Ca^{2+} influx even in the presence of a sufficient concentration of extracellular K^+ to depolarize the plasma membrane. When cells were superfused in 25 mM K^+-Hanks' solution [vs. the physiological 4.5 mM (7)] then heated, $InsP_2$ and $InsP_3$ still increased (860 ± 30 and 230 ± 50%, respectively, compared with unheated levels), but the $InsP_1$ level remained unchanged. The data suggest that these increases are not associated with membrane depolarization.

The functions of $InsP_1$ and $InsP_2$ are not known, but $InsP_3$ functions to mobilize Ca^{2+} from intracellular Ca^{2+} pools. For these reasons, we concentrated on the role of $InsP_3$ in the remainder of this study.

Effect of intracellular acidification on $InsP_3$. Because heat shock induces an intracellular acidification in A-431 cells, we determined whether intracellular acidification is associated with the heat-induced increase in $InsP_3$. When the intracellular pH (pH_i) of cells was adjusted to 6.80 from the normal pH_i of 7.23 ± 0.02 (20), $InsP_3$ did not change significantly (0.40 ± 0.03%total cpm, $n = 3$, $P = 0.05$) compared with values measured at the normal pH_i (0.38 ± 0.03%total cpm, $n = 3$). This indicates that the heat-induced intracellular acidification is not related to the heat-induced increase in $InsP_3$.

Effect of PLC on $InsP_3$. It is known that $[Ca^{2+}]_i$ concentrations over the range of 100 nM to 1 μM activate PLC, leading to the formation of $InsP_3$ (24). Heating increased $[Ca^{2+}]_i$ from 77 ± 6 to 223 ± 4 nM ($n = 3$), a concentration sufficient to activate PLC. We sought to determine what role PLC plays in the increase in $InsP_3$ stimulated by heat. If PLC activity were maximized and heating of the cells produced no additional increase in $InsP_3$ levels, then the heat-induced increase in $InsP_3$ would be consistent with an involvement of PLC. We stimulated PLC in unheated cells with the Ca^{2+} ionophore ionomycin (10 μM, 10 min; Ref. 31) in the presence of extracellular Ca^{2+}. Ionomycin activates PLC directly by elevating $[Ca^{2+}]_i$ (31). $InsP_3$ increased from 0.45 ± 0.10 to 1.22 ± 0.18%total cpm ($P < 0.05$, $n = 5$) in ionomycin-treated cells. Heating these cells (45°C, 20 min) did not promote an additional increase in $InsP_3$ (1.07 ± 0.11%total cpm, $n = 5$), which suggests heat stimulates an increase in $InsP_3$ through an activation of PLC.

Role of G proteins. PLC activity is coupled with G proteins (4). We sought therefore to monitor the relationship between G proteins and the increase in $InsP_3$ stimulated by heat. In the first experiment, the nonhydrolyzable GTP analogue Gpp(NH)p (10 μM), which prolongs G protein activity, was added to the cell homogenate 30 min before heating. In unheated cells, Gpp(NH)p stimulated an increase in $InsP_3$ of 57 ± 19% ($n = 3$, $P < 0.05$) as compared with those levels in cells neither heated nor treated with Gpp(NH)p. Heating the Gpp(NH)p-treated cells promoted an additional 31 ± 5% ($n = 3$, $P < 0.05$) increase in $InsP_3$. These values compare with an increase in $InsP_3$ of 89% in cells that were only heated (see Table 3), a value that represents the sum of both the G protein-stimulated increase in $InsP_3$ and the increase due to other heat-induced mechanisms, presumably a rise in $[Ca^{2+}]_i$ and cAMP. These experiments demonstrate not only the involvement of G proteins in the $InsP_3$ increase stimulated by heat but also the relative contributions of other factors in the process.

U-73122, an aminosteroid shown to block guanosine 5′-*O*-(3-thiotriphosphate)-stimulated $InsP_3$ production (28, 31) and thyrotropin-stimulated $InsP_3$ (27), also provides evidence of the involvement of G proteins and PLC. Treatment of unheated cells with 0.1–1 μM U-73122 for 20 min did not significantly affect the $InsP_3$ baseline; 5 μM U-73122 increased $InsP_3$ by only 19 ± 11% ($n = 3$). Heating the cells in the presence of U-73122 (0.5–5 μM) inhibited the heat-induced increase in $InsP_3$ in a concentration-dependent manner (Fig. 5), supporting the idea that a PLC-mediated process is involved in the response of the cells to heat.

Because $InsP_3$ is known to mobilize Ca^{2+} from intracellular pools, it would be expected that the reduced levels of $InsP_3$ in heated cells incubated with U-73122 should prevent the heat-induced $[Ca^{2+}]_i$ increase normally caused by $InsP_3$. The data summarized in Table 4 show that such was the case. Cells heated (45°C, 20 min) in the presence of external Ca^{2+} (1.6 mM) without U-73122 showed an increase in $[Ca^{2+}]_i$ from 77 ± 6 nM ($n = 27$) to 223 ± 4 nM ($n = 3$). Treatment of unheated cells with low concentrations of U-73122 (0.1 μM) slightly increased the resting $[Ca^{2+}]_i$ but did not block the increase in $[Ca^{2+}]_i$ that occurred when cells were heated (Table 4). However, higher concentrations of U-73122 (1 μM) inhibited the heat-induced increase by 37%, which further supports our previous observation (18). An inactive analogue of U-73122, U-73343, was tested to confirm the specificity of the inhibition produced by U-73122. U-73343 up to a concentration of 5 μM increased the resting $[Ca^{2+}]_i$ by 26% in unheated cells but had no effect on the increases in $[Ca^{2+}]_i$ (Table 4) or $InsP_3$ production observed after heating (data not shown), indicating that the inhibitory activity of U-73122 is specific.

We have previously shown that heat stimulates G proteins to increase cellular cAMP content (21). In this study we performed a series of experiments to modulate G protein activity and cAMP levels in the cell to determine the relationship between cAMP, G proteins, and the increase in $InsP_3$ observed after heating. We treated cells with pertussis toxin (PTX), an inhibitor of G inhibitory (G_i) protein, then exposed the cells to heat. PTX (30 ng/ml, 24 h) increased the basal level of $InsP_3$ in unheated cells, and heat treatment did not increase $InsP_3$ further, which suggests that the $InsP_3$ response to heat involves a PTX-sensitive G protein. This observation was not the result of PTX-induced changes in cAMP, because cAMP levels do not change after PTX treatment (21). Cholera toxin (CTX; 1 μg/ml, 1 h), an agonist of G

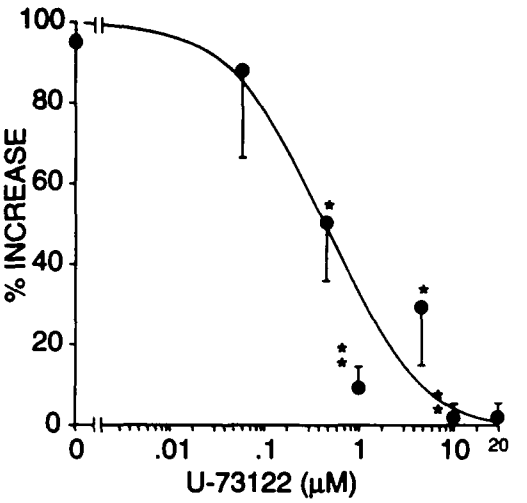

Fig. 5. U-73122 inhibition of heat-induced increase in $InsP_3$. Cells were exposed to either 37 or 45°C for 20 min in the presence of different concentrations of U-73122 ($n = 3$). Ordinate represents percentage changes of $InsP_3$ in heated cells in the presence of indicated concentrations of U-73122. * $P < 0.05$ vs. untreated cells; ** $P < 0.05$ vs. untreated cells and cells treated with 0.01, 0.1, 0.5, or 5 μM U-73122.

Table 3. *TMB-8 did not affect heat-induced $InsP_3$ increase in presence of external Ca^{2+}*

Treatment	$InsP_3$, % total cpm	
	Unheated	Heated
$+Ca_e^{2+}$	0.99±0.11	1.87±0.33*
$-Ca_e^{2+}$	0.81±0.03	0.78±0.08
$+Ca_e^{2+}$ + TMB-8	1.12±0.11	1.99±0.08*
$-Ca_e^{2+}$ + TMB-8	0.38±0.10	0.46±0.10

Values are means ± SE and are expressed as a percentage of total radioactivity (cpm, counts per minute) in cells at time of inositol metabolite extraction. Cells were exposed to 45°C for 20 min in presence of 3,4,5-trimethoxybenzoic acid 8-(diethylamino)octyl ester (TMB-8; 100 μM) with 1.6 mM external Ca^{2+} (Ca_e^{2+}) or without external Ca^{2+} ($n = 3$ or 4). * $P < 0.05$ vs. unheated cells.

Table 4. *U-73122 but not U-73343 attenuated heat-induced increase in $[Ca^{2+}]_i$*

Treatment	Concentration, μM	$[Ca^{2+}]_i$, nM	
		Unheated	Heated
Control	0	77±6	223±4‡
U-73122	0.1	105±4*	241±26‡
	1	90±18*	141±13†
U-73343	5	97±3*	225±6‡

Values are means ± SE. $[Ca^{2+}]_i$, cytosolic free Ca^{2+} concentration. Cells were exposed to 45°C for 20 min in presence of either chemical and 1.6 mM external Ca^{2+} ($n = 3$–24). * $P < 0.05$ vs. unheated control cells. † $P < 0.05$ vs. unheated cells treated with 1 μM U-73122 and heat-treated cells. ‡ $P < 0.05$ vs. unheated cells and heated U-73122 (1 μM)-treated cells.

stimulatory (G_s) protein, also increased the basal level of $InsP_3$, and, like the results after PTX treatment, $InsP_3$ did not increase further after heating (Fig. 6). Because CTX also elevates cAMP due to G_s activation, the increase in $InsP_3$ may be the result of either a direct stimulation of G_s or an increase in cAMP level. To distinguish between these two possibilities, we incubated cells with forskolin, an adenylate cyclase stimulator, which we have shown increases intracellular cAMP (21). Forskolin (150 μM, 10 min; Refs. 12, 21, 22) also increased $InsP_3$ (Fig. 6), which indicates there is a possible relationship between cAMP and $InsP_3$.

Effect of cAMP on $InsP_3$. If the increase in $InsP_3$ induced by heat were the result of a heat-induced increase in cAMP, then treatment of cells with cAMP analogues should stimulate $InsP_3$ production. In unheated cells the basal level of cAMP is 2,086 ± 139 fmol/10^6 cells; heating at 45°C increases cAMP to 3,087 ± 142 fmol/10^6 cells (21). Table 5 shows that treatment of unheated cells with 8-BrcAMP (1 mM) elevated $InsP_3$ to the levels seen in heated cells. The phosphodiesterase inhibitors IBMX (1 mM), Ro 20-1724 (0.5 mM), or theophylline (1 mM) also increased the resting $InsP_3$ in unheated cells to levels similar to those measured after exposure to heat. Heat treatment in the presence of each of these agents did not promote a further increase in $InsP_3$ (Table 5), indicating that the heat response and the cAMP response share similar pathways.

There is a direct relationship between Ca^{2+} and cAMP in promoting the increase in $InsP_3$. Cells treated with 8-BrcAMP (1 mM, 10 min) in the presence of extracellular Ca^{2+} had higher $InsP_3$ levels than untreated controls (Table 5). Cells treated with 8-BrcAMP in the absence of extracellular Ca^{2+} failed to demonstrate the increase in $InsP_3$ seen when extracellular Ca^{2+} was present (in %total cpm:control, 0.29 ± 0.01; 8-BrcAMP, 0.33 ± 0.03; n = 3, $P > 0.05$). Likewise, treatment with verapamil (1 mM) blocked the increase in $InsP_3$ stimulated by 8-BrcAMP (in %total cpm:control, 1.73 ± 0.17; 8-BrcAMP, 2.10 ± 0.02; verapamil, 1.72 ± 0.14; verapamil + 8-BrcAMP, 1.52 ± 0.11; n = 4 for each group). 8-BrcAMP did not increase $InsP_3$ further when cells were pretreated with ionomycin (1 μM).

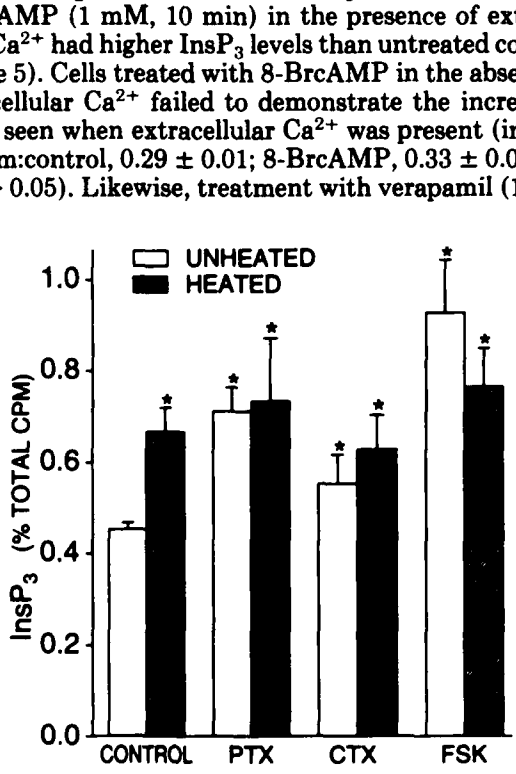

Fig. 6. Effect of pertussis toxin (PTX), cholera toxin (CTX), or forskolin (FSK) on heat-induced increase in $InsP_3$. Cells were pretreated with PTX (30 ng/ml, 24 h), CTX (1 μg/ml, 1 h), or FSK (150 μM, 10 min) then exposed to 45°C for 10 min (n = 3). Data are expressed as a percentage of total radioactivity in cells at time of inositol metabolite extraction. * $P < 0.05$ vs. unheated control cells.

Table 5. *Effect of a cAMP analogue and phosphodiesterase inhibitors on heat-induced increase in $InsP_3$*

Agent	Concentration, mM	$InsP_3$, % total cpm	
		Unheated	Heated
Control	0	0.58±0.04	0.77±0.02*
8-BrcAMP	1	0.93±0.05*	0.83±0.07*
IBMX	1	0.76±0.05*	0.67±0.11*
Ro 20-1724	0.5	0.81±0.02*	0.67±0.05*
Theophylline	1	0.84±0.05*	0.83±0.05*

Values are means ± SE and are expressed as a percentage of total radioactivity in cells at time of inositol metabolite extraction. 8-BrcAMP, 8-bromoadenosine 3',5'-cyclic monophosphate; IBMX, 3-isobutyl-1-methylxanthine. Cells were treated with these agents at either 37 (control) or 45°C for 10 min (n = 3-6). * $P < 0.05$ vs. unheated control cells.

The increase in $InsP_3$ levels that results from increasing the cAMP correlates with increases in $[Ca^{2+}]_i$. cAMP has been shown to increase the opening probability of L-type Ca^{2+} channels as seen in cardiac myocytes. A 10-min incubation with 8-BrcAMP (1 mM) increased $[Ca^{2+}]_i$ 35 ± 5% (n = 4). The increase was abolished in the absence of extracellular Ca^{2+}, suggesting that the 8-BrcAMP-induced increase was due to an increase in Ca^{2+} influx. Furthermore, this increase was inhibited by treatment with La^{3+} (1 mM) or verapamil (1 mM; data not shown), indicating voltage-gated Ca^{2+} channels were involved. These data, taken together with the fact that verapamil blocked the increase in $InsP_3$, suggest that the cAMP-induced increase in $InsP_3$ is a result of an increase in $[Ca^{2+}]_i$.

DISCUSSION

This study demonstrates that heat shock produced an increase in inositol phosphates in human epidermoid carcinoma A-431 cells that was related to both temperature and the duration of heating. Inositol phosphate levels in heated cells returned to normal within 15 min after heating. To investigate the mechanisms underlying the heat-induced increase in $InsP_3$, we examined the roles of extracellular Ca^{2+}, PLC-mediated processes, and G protein interactions in this process. Our results show that the heat-stimulated uptake of Ca^{2+} and increase in cAMP, the stimulation of PLC by Ca^{2+}, and the activation of G regulatory proteins all contributed to the heat-induced increase in $InsP_3$.

We showed in a previous study that heat treatment causes a maximal influx of Ca^{2+} within 10 min (18). The primary mechanism for this Ca^{2+} entry is through Na^+-Ca^{2+} exchange. The maximal increase in $InsP_3$ occurred 20 min after heating, which is consistent with the hypothesis that the heat-induced increase in $InsP_3$ is a consequence of the increase in $[Ca^{2+}]_i$ due to a Ca^{2+} influx. The observation that there was no change in $[Ca^{2+}]_i$ and

$InsP_3$ in the absence of extracellular Ca^{2+} or in the presence of amiloride supports this view. PLC probably mediates this increase in $InsP_3$ because it is the only Ca^{2+}-sensitive component of the $InsP_3$ pathway. The facts that ionomycin stimulated PLC activity and that PLC activity did not increase further in ionomycin-treated cells after heating indicate that PLC plays a role in the $InsP_3$ heat response.

Data obtained from Gpp(NH)p and U-73122 experiments indicate an involvement of G proteins. Gpp(NH)p, which potentiates G protein activity, stimulates an increase in $InsP_3$ in unheated cells that is increased further when those cells are heated. However, the sum of those two increases is equivalent to that seen in cells heated in the absence of Gpp(NH)p. Gpp(NH)p treatment therefore mimics that portion of the heat-induced increase in $InsP_3$ due to G protein activation, with the remainder of the increase in $InsP_3$ being derived from the increases in $[Ca^{2+}]_i$ and cAMP that are also stimulated by heat. The heat-induced $InsP_3$ increase was inhibited by U-73122, an inhibitor of PLC-mediated processes. The $InsP_3$ response to U-73122 observed in our experiments was similar to that observed in several other kinds of cells. The inhibitory dissociation constant for U-73122 in human platelets is 9–40 μM, in human polymorphonuclear neutrophils it is 2 μM (6), and in rat pituitary cells it is 3.5 μM (27). But at a concentration <1 μM, U-73122 completely inhibits formyl-methionyl-leucyl-phenylalanine (FMLP)-stimulated granule exocytosis and superoxide anion production in human polymorphonuclear neutrophils (28, 31). Therefore, the observation that this compound at micromolar concentrations effectively inhibited the heat-induced increase in $InsP_3$ in A-431 cells was not surprising. It is worth noting that at a concentration >5 μM U-73122 itself promoted Ca^{2+} influx and $InsP_3$ production in A-431 cells. Similar observations have been made for rat pituitary cells (27).

Our experiments with PTX suggest that PTX-sensitive G proteins are involved in increasing $InsP_3$. Both PTX and CTX elevated basal levels of $InsP_3$ in unheated cells. Heating, however, did not promote an additional increase. A similar finding was reported in FMLP-stimulated leukocytes (7, 32). The data that PTX treatment increased $InsP_3$ levels without altering cAMP levels suggest that this PTX-sensitive G protein is different from G_i. Other evidence has accumulated that there is a G protein (G_p) distinct from G_i and G_s (24). It is possible that this PTX-sensitive G protein is G_p. Heat shock may directly stimulate the PTX-sensitive G protein as heat does with the Na^+-Ca^{2+} exchanger, increasing the exchanger's affinity for binding to external Ca^{2+} (18).

Previously, this laboratory had reported that G proteins are involved in the heat-induced increase in cellular cAMP content. The data presented in this study suggest that the mechanism leading to an $InsP_3$ increase may be the same as that involved in the cAMP increase, because treatment with CTX or forskolin affected both the $InsP_3$ and cAMP heat response. 8-BrcAMP and agents that increase intracellular cAMP increased the resting level of $InsP_3$. This is consistent with PTX and CTX effects and is in agreement with the observations of Pike and Eakes (26). The difference between our data and data from rat neutrophils (15) and rat kidney slices (25) is likely due to the different cells employed for the study. It is worth noting that Mahe et al. (22) reported that 100 μM forskolin increased Na^+ uptake and cell volume in fish erythrocytes in addition to stimulating adenylate cyclase activity. More studies are needed to determine if A-431 cells respond in a way similar to forskolin and, if so, if the increase in Na^+ uptake and cell volume causes changes in $InsP_3$.

We found that the cAMP-induced increase in $InsP_3$ was dependent on external Ca^{2+}. The observation that $InsP_3$ did not further increase after heating cells treated with agents that increased intracellular cAMP supports the view that the heat response and the cAMP response share the same pathways. Three observations are consistent with the idea that the cAMP-induced increase in $InsP_3$ is due to the activation of PLC via a Ca^{2+} influx: *1*) removal of extracellular Ca^{2+} prevented the cAMP-induced increase in $[Ca^{2+}]_i$ and $InsP_3$; *2*) both increases were blocked in the presence of verapamil, a voltage-gated Ca^{2+} channel blocker; and *3*) treatment with 8-BrcAMP in the presence of ionomycin did not further increase $InsP_3$ production.

A proposed mechanism for the heat-induced $InsP_3$ increase is shown in Fig. 7. Heat activates the reversed mode of Na^+-Ca^{2+} exchange so as to increase $[Ca^{2+}]_i$ (18). Heat also increases intracellular cAMP via activation of G_s protein and adenylate cyclase (21). The cAMP increase activates protein kinase A that phosphorylates verapamil-sensitive Ca^{2+} channels, which results in the

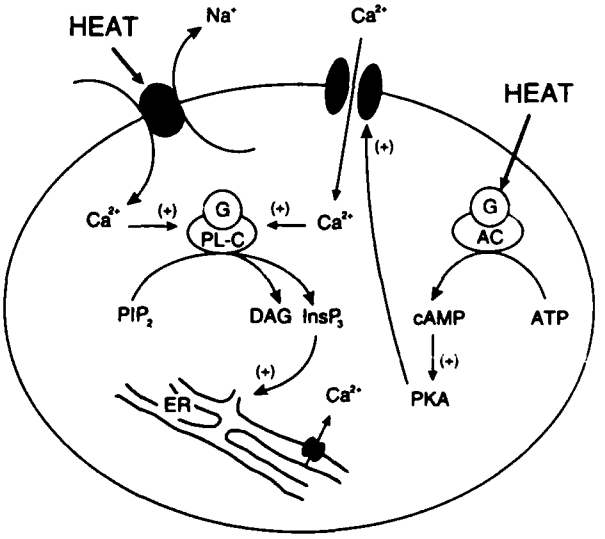

Fig. 7. Proposed mechanism of heat-induced $InsP_3$ increase. Heat activates reversed mode of Na^+-Ca^{2+} exchange that results in an increase in cytosolic free Ca^{2+} concentration ($[Ca^{2+}]_i$). Then, the increase in $[Ca^{2+}]_i$ triggers phospholipase C (PLC) activity, thereby increasing $InsP_3$ and diacylglycerol (DAG) levels. Heat also stimulates G protein and adenylate cyclase (AC), which increase cAMP production. The resulting increase in cAMP activates protein kinase A (PKA), which in turn phosphorylates Ca^{2+} channels. Increased Ca^{2+} influx through Ca^{2+} channels elevates intracellular Ca^{2+} levels that promote PLC activity. PIP_2, phosphatidylinositol 4,5-bisphosphate; ER, endoplasmic reticulum; (+), stimulated activities.

influx of extracellular Ca^{2+}. This Ca^{2+} increase then triggers PLC activity and activation of PTX-sensitive G protein, which results in increased $InsP_3$ production.

Most of the metabolic consequences of an increase in $InsP_3$ promoted by heat remain to be examined. However, a known consequence is an increase in $[Ca^{2+}]_i$ resulting from the mobilization of intracellular Ca^{2+}, because such a mobilization can be blocked by the $InsP_3$ production inhibitor U-73122. This transient increase in $[Ca^{2+}]_i$ due to a transient increase in $InsP_3$ may be important for cell regeneration because Birch et al. (5) reported that in adult murine neuron a Ca^{2+} transient increase was responsible for axonal regeneration. In our lab we have observed a synthesis of the 70-kDa heat shock protein mRNA beginning 30 min after heating that is correlated with the return of $InsP_3$ to basal levels (J. G. Kiang, F. E. Carr, and D. E. McClain, unpublished observations). Expression of the 70-kDa heat shock protein is attenuated by treatment with U-73122, an inhibitor of $InsP_3$ production (19). Because $InsP_3$ freely diffuses throughout the cytoplasm, it may play a very important role in controlling Ca^{2+} homeostasis (14). It would be interesting to determine whether an increase in $InsP_3$ is one of the reconstructive mechanisms that allows cells to recover from heat-induced dysfunction.

In summary, heat treatment increases $InsP_1$, $InsP_2$, and $InsP_3$. The increases are temperature dependent. The heat-induced increase in $InsP_3$ does not occur in the absence of extracellular Ca^{2+} or in the presence of amiloride or U-73122. PTX, CTX, 8-BrcAMP, or cAMP-increasing agents stimulate an increase in $InsP_3$ in unheated cells that does not increase further after heat treatment. These results show that heat stimulates an increase in $[Ca^{2+}]_i$ and PTX-sensitive G proteins that cause an increase in $InsP_3$. Heat also stimulates cAMP that mediates an additional increase in $[Ca^{2+}]_i$ that adds to the effect on $InsP_3$.

We thank Dr. S. M. Aloj for critical suggestions. We also thank Lisa Drouin for technical assistance.

This work was supported by the Armed Forces Radiobiology Research Institute, Defense Nuclear Agency Work Units 00020 and 00150, and Walter Reed Army Institute of Research, Department of the Army In-house Laboratory Independent Research Fund ECN 0027. Views presented in this paper are those of the authors; no endorsement by the Defense Nuclear Agency or Department of the Army has been given or should be inferred.

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Received 30 September 1992; accepted in final form 26 January 1993.

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